Immunology 1985 **54** 513

Autoreactive T-cell lines specific for mouse thyroglobulin

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Accepted for publication 29 October 1984

Summary. Autoreactive T-cell lines specific for mouse thyroglobulin have been established and characterized. These Lyt 1^+ T cells proliferated specifically in response to thyroglobulin presented by syngeneic irradiated spleen cells. The antigen-presenting cell requirements of these autoreactive T cells appeared to be the same as those for foreign antigen (PPD) reactive T cells. All lines tested required antigen-presenting cells compatible at the I-A subregion of the H-2 complex. Both T-cell types responded to antigen presented by peritoneal cells and splenic dendritic cells, but only gave optimal responses when whole spleen cells were used.

The cross-reactivity patterns of responses to mouse, rat, pig and human thyroglobulins indicated that at least two different epitopes could be recognized by the autoreactive T cells. Furthermore, these epitopes appeared to be different from those recognized by the majority of serum autoantibodies to mouse thyroglobulin.

INTRODUCTION

Experimental autoimmune thyroiditis and thyroglobulin-specific autoantibodies can be induced in mice by immunizing them with mouse thyroglobulin (Tg), with or without various adjuvants (Elrehewy *et al.*, 1981; Rose, Twarog & Crowle, 1971). Both thyroid-specific cytotoxic T cells and autoreactive T helper cells may be involved in the autoimmune processes (Creemers, Rose & Kong, 1983; Rose *et al.*, 1981). Furthermore, thyroglobulin-specific suppressor T cells capable of down-regulating the autoimmune responses have been shown to be induced by appropriate immunization protocols (Kong *et al.*, 1982). We are attempting to dissect the regulatory mechanisms involved in the autoimmune responses to thyroglobulin by raising autoantigen-specific T-cell lines and clones. This approach, using cell lines reactive to myelin basic protein, has already provided interesting insights into the regulation of experimental autoimmune encephalomyelitis (Naparstek *et al.*, 1983; Ben-Nun & Cohen, 1981). We report here the characterization of thyroglobulin-specific autoreactive T-cell lines and have compared the presentation of self antigen to these cells with the presentation of foreign antigen to PPD-reactive cell lines.

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MATERIALS AND METHODS

Mice

Male CBA/Ca, B10.Br, BALB/c, C57BL and SJL mice were obtained from the MRC laboratories, Mill Hill, London. Recombinant inbred mice B10.A, ATL, B10.HTT, B10.A (3R), B10.A (4R) and B10.A (5R) were obtained from OLAC.

Antigens

Mouse, rat, pig and human thyroglobulins (Tg) were

prepared by overnight extraction of homogenized thyroid tissues in PBS, followed by ultracentrifugation, differential ammonium sulphate precipitation and Sepharose 6B chromatography as previously described (De Carvalho, Wick & Roitt, 1980). Purified protein derivative of tuberculin (PPD) was obtained from the Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey.

T-cell growth factor (TCGF)

Pooled rat spleen cells (10^7/ml) were cultured with Concanavalin A (2·5 μg/ml) in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS), 10^{-5} M 2-mercaptoethanol, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). After 30 hr, the cells were removed by centrifugation, and Concanavalin A in the supernatant was blocked by the addition of excess α-D-methylmannoside (20 mg/ml). The supernatant was sterilized by 0·2 μm filtration and stored frozen until used at the optimal concentration of 12·5% (v/v).

T-cell lines

CBA/Ca mice were immunized in the hind footpad or intraperitoneally with mouse Tg (50 μg) emulsified in Freund's complete adjuvant, containing heat-killed *Mycobacterium tuberculosis* (1 mg/ml) (Difco Laboratories, Detroit, MI). Seven or eight days later, draining popliteal lymph nodes or spleens were removed and cell suspensions prepared by teasing with forceps. After three washes with BSS, cells were cultured in Falcon 50-ml flasks at 10^7/ml in DMEM/10% FCS (Dulbecco's modified Eagle medium supplemented with 10% (v/v) FCS, 1% (v/v) non-essential amino acids (Flow Laboratories, Irvine, Scotland), 110 μg/ml sodium pyruvate, 10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 20 μg/ml gentomycin) in the presence of mouse Tg (100 μg/ml) or PPD (50 μg/ml). The cells were cultured at 37° in a humidified atmosphere of 5% CO_2 in air. Cells were restimulated every 7–8 days with mouse Tg (50 μg/ml) or PPD (25 μg/ml) and 2×10^7 irradiated (2000 rads) syngeneic spleen cells, in a total volume of 8 ml. Flasks were always cultured upright to promote cell-cell contact for antigen stimulation. Fresh medium (2·5 ml) was added 3 or 4 days after restimulation. A cell concentration of $2–4 \times 10^5$/ml ($1·5–3 \times 10^6$ cells/flask) at the time of restimulation appeared to give optimal growth conditions, as assessed by the yield of viable cells 7–8 days later. Cells would usually survive no longer than 10–12 days without being restimulated with antigen. After 2–3 months of growth, TCGF (12·5–15%) was added to the culture medium.

Proliferation assay

Line cells were washed once with BSS and adjusted to 2×10^5/ml in DMEM/10% FCS. Triplicate cultures were established in 96-well flat-bottom microtitre plates (Sterilin, Teddington, Middlesex) in a total volume of 200 μl containing 2×10^4 line cells, an appropriate concentration of antigen-presenting cells (optimally 10^6 spleen cells, 5×10^4 peritoneal cells, 5×10^4 dendritic cells) and various concentrations of test antigens. Cells were cultured for 3 days with ^{125}I-deoxyuridine (0·5 μCi) added for the final 18 hr of culture. Cultures were then harvested onto glass fibre discs with a Titertek cell harvester (Flow Laboratories) and incorporated radiolabel assessed by gamma counting.

Antigen-presenting cells

Spleen cells were prepared by teasing in BSS, washed three times and then adjusted to 10^8/ml in DMEM/10% FCS. Peritoneal lavage cells were obtained by washing the peritoneal cavity of normal mice with 5 ml BSS. Cells were washed three times with BSS and adjusted to 10^6/ml in DMEM/10% FCS. Dendritic cells were prepared from spleen cells as previously described (Sunshine, Katz & Feldmann, 1980). Briefly, low buoyant density cells separated by discontinuous density gradient centrifugation were further purified by differential adherence after 2 hr and 18 hr culture. The non-adherent cells at 18 hr were further fractionated by removal of Fc receptor positive cells using a rosetting technique. Dendritic cells produced by this method were non-phagocytic, Ia-positive and Fc receptor negative (Sunshine *et al.*, 1980). Data are only presented for optimal antigen-presenting cell numbers which were: 10^6 spleen cells, 5×10^4 dendritic cells and 5×10^4 peritoneal cells.

RESULTS

Characterization of cell lines

Several long-term T-cell lines reactive with mouse thyroglobulin or PPD have been established by a process of repeated *in vitro* stimulation with antigen in the absence of added growth factors. Some of these lines have been in continuous culture for up to a year. The cells are of the Thy 1^+, Ly 1^+, 2^-, Ig^- phenotype (data not shown) and respond in proliferation assays

Table 1. Specificity of T-cell lines

Cell line	Proliferative response* No antigen	MTg	PPD
Mouse thyroglobulin lines			
MTg7	1·4±0·3	15·8±1·0	1·4±0·1
MTg8	0·4±0·1	7·2±0·5	0·5±0·2
MTg9	7·9±1·2	13·4±0·6	4·2±0·3
MTg12	0·8±0·1	13·3±1·2	1·2±0·4
PPD lines			
PPD6	2·6±0·3	2·3±0·4	10·1±0·7
PPD7	2·5±0·4	1·9±0·3	32·3±0·8
PPD9	0·9±0·2	0·7±0·2	9·9±1·1

Triplicates of 2×10^4 line cells and 10^6 irradiated (2000 rads) CBA/Ca spleen cells were cultured (200 μl total volume) in 96-well microtitre plates in the presence or absence of mouse thyroglobulin (MTg; 50 μg/ml) or PPD (25 μg/ml) as antigens. ^{125}I-deoxyuridine (0·5 μCi) was added to each well for the final 16 hr before the cells were harvested onto glass fibre discs and counted.

* Incorporation of ^{125}I-deoxyuridine (c.p.m. $\pm$SE $\times 10^{-3}$).

only when the appropriate selecting antigen is present (Table 1).

Cross-reactivity

Most of the mouse Tg-specific lines responded as well or better to rat or human Tg, but only very poorly to pig Tg (shown for MTg7 in Fig. 1a). However, one cell line (MTg12) responded poorly to human Tg, but equally well to pig and rat Tg (Fig. 1b). Both cross-reactivity patterns contrast with that shown by serum antibodies from mice immunized with Tg in CFA (Fig. 1c). The strength of reactivity of such antisera is consistently in the order: mouse Tg > rat Tg > pig and human Tg. Furthermore, although most murine monoclonal autoantibodies to Tg also react with rat Tg, only a few react with human Tg (our unpublished observations, and Rose *et al.*, 1982).

H-2 restriction of the proliferative responses

The proliferative responses of the cell lines to specific antigen were only obtained when the antigen was

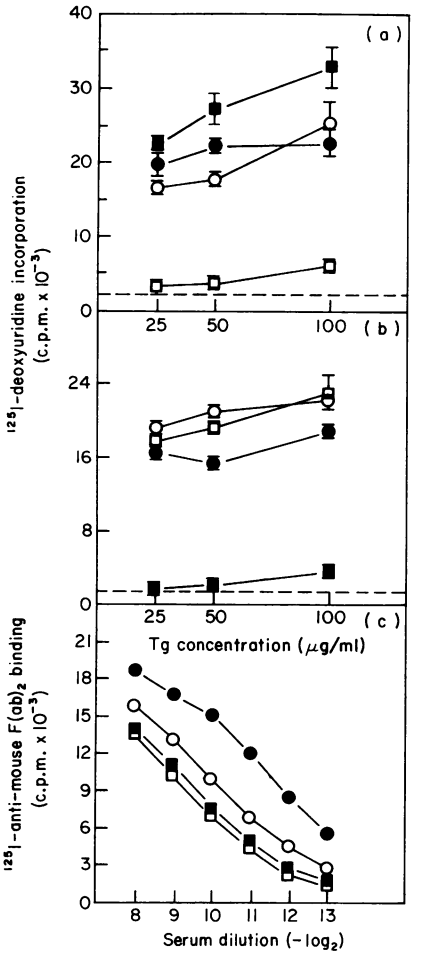

Fig. 1. Cross-reactivity patterns of autoantigenic epitopes on mouse thyroglobulin showing proliferative responses of (a) MTg7 and (b) MTg12 T-cell lines to mouse (●——●), rat (○——○), pig (□——□) and human (■——■) thyroglobulins. T cells (2×10^4) were cultured with syngeneic irradiated spleen cells (10^6) in triplicate 200-μl cultures in flat-bottomed microtitre plates in the presence of various concentrations of thyroglobulins. After 48 hr culture, the cells were pulsed with 0·5 μCi ^{125}I-deoxyuridine and harvested and counted 15 hr later. The level of incorporation of radiolabel into unstimulated cells is represented by the dashed lines (mean c.p.m. $\pm$ 1 SE). (c) reactivity of serum thyroglobulin autoantibodies with different thyroglobulins. Binding of serum autoantibodies (from mice immunized with mouse thyroglobulin in Freund's complete adjuvant) at various dilutions to solid phase mouse (●——●), rat (○——○), pig (□——□) and human (■——■) thyroglobulin was detected with a ^{125}I-labelled affinity-purified sheep anti-mouse F(ab)$_2$ antibody.

Table 2. H-2 restriction of proliferative responses of T-cell lines

Strain of APC	H-2	Antigen*	Proliferative responses† MTg7	MTg8	PPD6	PPD7
CBA/Ca	*k*	–	1·1±0·2	1·0±0·3	0·7±0·2	0·7±0·1
		+	22·4±2·0	15·2±0·6	13·3±0·2	10·1±1·9
C3H	*k*	–	0·6±0·1	0·8±0·2	0·2±0·1	1·6±0·5
		+	9·6±0·8	6·2±0·9	11·6±0·8	6·4±0·2
BALB/c	*d*	–	0·9±0·2	1·0±0·2	0·3±0·1	0·4±0·1
		+	0·7±0·2	0·9±0·2	0·3±0·1	0·5±0·1
SJL	*s*	–	2·3±0·3	NT‡	0·5±0·1	NT
		+	2·2±0·2	NT	0·7±0·2	NT
C57BL	*b*	–	1·0±1·0	NT	0·2±0·0	NT
		+	1·0±0·1	NT	0·3±0·1	NT
(CBA × BALB/c)F_1	*k/d*	–	1·0+0·3	1·0±0·1	1·6±0·4	NT
		+	10·8±1·5	8·0±0·9	8·4±0·6	NT

Proliferation assays were set up as described in Table 1 using spleen cells from different mouse strains as antigen-presenting cells (APC).

* Mouse Tg-specific lines (MTg7 and MTg8) were cultured with or without 50 μg/ml mouse Tg; PPD-specific lines (PPD6 and PPD7) with or without 25 μg/ml PPD.

† Proliferation expressed as c.p.m. of incorporated ^{125}I-deoxyuridine ± SE × 10^{-3}.

‡ NT, not tested.

presented by irradiated spleen cells of the appropriate H-2 haplotype (H-2^k or H-$2^{k/d}$) (Table 2). Responses with (CBA × BALB/c)F_1 spleen cells were always markedly lower than with CBA spleen cells, but the level of responses to C3H (H-2^k) spleen cells varied from experiment to experiment. These observations most likely reflect differing levels of expression of restricting elements (Ia antigens) on the spleen cells (Conrad *et al.*, 1982), perhaps caused by differing environmental conditions of the mice. By using spleen cells prepared from recombinant inbred mouse strains, the presentation of both Tg and PPD to the cell lines has been shown to require compatibility at the I-A subregion of the H-2 complex (Table 3).

Lack of influence of the Mls locus

Janeway *et al.* (1983) recently reported an influence of Mls locus (mixed lymphocyte stimulation response locus on chromosome 1) alleles on proliferative responses of T cells. We have tested cells from various H-2^k strains of mice which differed at the Mls locus as antigen-presenting cells. The results showed no apparent effect of the Mls locus on the responses of either the mouse Tg or PPD specific lines (data not shown).

Antigen-presenting cells

The specificity and I-A restriction of the cell lines were carried out using whole spleen cells as a source of antigen-presenting cells. Since both macrophages and dendritic cells have been shown to have antigen-presenting function (Unanue *et al.*, 1984; Steinman & Nussenzweig, 1980), and different presenting cells may stimulate different T-cell subsets (Ramila & Erb, 1983), we studied the ability of splenic dendritic cells and peritoneal lavage cells to present autoantigen (MTg) and foreign antigen (PPD) to the cell lines. The results in Table 4 demonstrate that dendritic cells could present both MTg and PPD to the cell lines. However, the responses were generally much lower than those obtained with whole spleen cells presenting antigen, particularly for the thyroglobulin-specific lines. One exception to this was the PPD9 cell line which, in one experiment, failed to respond to PPD with unseparated spleen cells, but did respond with purified dendritic cells as antigen presenters. Resident peritoneal cells were also capable of presenting antigen to both PPD and MTg specific lines but again, in most instances, the responses were markedly lower than those obtained with spleen cells. The proliferative responses could be improved markedly if the peri-

Table 3. I-A restriction of proliferative responses of T-cell lines

Mouse strain of APCs	H-2 K	A	E	D	Antigen*	Proliferative responses† MTg7	MTg9	PPD6	PPD7
CBA/Ca	*k*	*k*	*k*	*k*	–	0·7±0·0	4·6±0·3	1·0±0·1	0·7±0·3
					+	17·3±1·8	16·6±0·9	19·2±1·1	7·7±0·5
B10.BR	*k*	*k*	*k*	*k*	–	0·6±0·0	2·5±0·1	2·6±0·4	0·8±0·0
					+	7·2±0·3	9·5±0·5	20·7±2·3	4·9±0·3
ATL	*s*	*k*	*k*	*d*	–	0·9±0·2	4·9±0·2	1·2±0·1	1·1±0·3
					+	10·5±0·6	17·8±0·7	36·9±0·6	6·3±0·6
B10.HTT	*s*	*s*	*k*	*d*	–	0·8±0·1	0·9±0·2	0·5±0·1	0·5±0·2
					+	0·9±0·1	0·3±0·1	1·1±0·3	0·6±0·1
B10.A	*k*	*k*	*k*	*d*	–	0·5±0·0	2·1±0·1	2·4±0·4	0·4±0·1
					+	6·3±0·8	7·9±0·6	16·1±1·0	3·6±0·1
B10.A (3R)	*b*	*b*	*k*	*d*	–	0·5±0·1	2·9±0·7	2·8±0·3	NT‡
					+	0·8±0·3	0·7±0·6	0·6±0·2	
B10.A (4R)	*k*	*k*	*b*	*b*	–	0·4±0·0	1·1±0·0	0·8±0·3	0·5±0·0
					+	5·4±0·1	5·4±0·9	15·6±0·8	3·7±0·1
B10.A (5R)	*b*	*b*	*k*	*d*	–	0·3±0·0	0·4±0·0	0·3±0·0	0·3±0·0
					+	0·4±0·1	0·6±0·3	0·6±0·1	0·4±0·0

Details and footnotes as for Table 2.

Table 4. Proliferative responses of lines to antigen presented by different cell types

	Line	Antigen*	Proliferative responses† stimulated by: Unseparated spleen cells	Dendritic cells	Peritoneal cells	Peritoneal cells (not irradiated)
Exp. 1	MTg12	–	0·9±0·4	0·7±0·0	0·3±0·1	0·7±0·1
		+	26·0±3·2	2·2±0·1	8·1±1·2	27·7±3·7
	PPD9	–	2·0±0·1	0·5±0·0	0·4±0·1	3·6±0·3
		+	2·2±0·1	6·0±0·6	12·7±0·6	28·6±0·9
Exp. 2	MTg7	–	1·2±0·1	0·9±0·3	NT‡	NT
		+	30·6±2·5	2·0±0·1		
	PPD6	–	9·8±1·0	0·3±0·0	NT	NT
		+	27·7±1·5	8·6±0·5		
Exp. 3	MTg7	–	0·9±0·1	0·5±0·2	NT	NT
		+	12·3±0·7	2·1±0·1		
	PPD7	–	0·4±0·1	0·2±0·0	NT	NT
		+	18·3±1·4	2·6±0·4		
Exp. 4	MTg7	–	0·7±0·0	NT	0·4±0·0	1·7±0·2
		+	17·3±1·8		2·1±0·2	14·2±1·3
	MTg9	–	4·6±0·2	NT	2·4±0·0	4·4±0·8
		+	16·6±1·0		5·6±0·1	9·6±0·8
	PPD6	–	1·0±0·1	NT	0·4±0·1	1·4±0·1
		+	19·2±1·1		6·6±0·2	8·3±0·6
	PPD7	–	0·7±0·3	NT	0·7±0·4	1·6±0·4
		+	7·7±0·5		3·5±0·1	18·0±2·5

The dendritic cells and peritoneal cells were used at 5×10^4 per well. The remaining details and footnotes are as for Table 2.

toneal cells were not irradiated prior to use in the assay (Table 4). This observation might reflect an inherent radiation-sensitivity of some presenting cells (Ashwell *et al.*, 1984), or could imply that T or B cells in the peritoneal cell preparations are being induced to proliferate in the presence of antigen-activated lines, since peritoneal cells themselves do not respond to thyroglobulin or PPD (data not shown).

DISCUSSION

Indirect evidence has indicated the existence of autoreactive T helper cells specific for thyroglobulin in normal mice (Rose *et al.*, 1981; Charreire, 1982). However, these experiments used heterogeneous lymphocyte populations. We have raised murine autoreactive T-cell lines specific for Tg using lymph node and spleen cells from animals immunized with mouse Tg. These cell proliferate specifically to Tg in the presence of I-A compatible antigen-presenting cells. Other experiments (Champion *et al.*, manuscript submitted) have shown that the cells also release IL-2 and B-cell helper factors following stimulation with Tg. These observations indicate that the cell lines consist of autoreactive T helper cells and provide direct evidence that such cells (at least those specific for Tg) are not deleted from the T-cell repertoire, but exist in normal mice. These cells are presumably under some, as yet uncharacterized, suppressive influence(s), since normal mice do not develop autoimmunity to thyroglobulin spontaneously. This may be the role of the thyroglobulin-specific T suppressor cells, which can be induced by some immunization protocols (Kong *et al.*, 1982).

Various Ia-positive cell types are capable of presenting antigens (Unanue *et al.*, 1984), and recent evidence suggests that different T-cell subsets may respond to antigen presented by different cells (Ramila & Erb, 1983). We were interested in investigating whether autoreactive T cells had different antigen presentation requirements from T cells reacting to conventional foreign antigens. The results reported here indicate that thyroglobulin-specific autoreactive T cells respond to autoantigen under the same conditions required by PPD-specific T cells. That is, both T-cell types required I-A compatible presenting cells and, although able to respond to antigen presented by peritoneal macrophages and dendritic cells, usually proliferated optimally with whole spleen cells as antigen-presenting cells. This might have been because the cell lines were grown using whole spleen cells to present antigen (Ramila & Erb, 1983), but could also suggest the possibility that the cell lines require both macrophages and dendritic cells for optimal responses. Alternatively, there may be two cell types present in a line; one responding to macrophages, and the other to dendritic cells as antigen presenters. It is also possible that functional activation of T cells, such as the release of lymphokines or helper factors, may require different antigen presentation from the proliferative responses. We are hoping to further investigate these possibilities using a panel of cloned T-cell populations. However, we have thus far only been able to establish one cloned thyroglobulin-specific cell line.

The two cross-reactivity patterns of the thyroglobulin-specific T-cell lines both differed markedly from that seen with serum autoantibodies to mouse Tg. This implies that there are at least two epitopes on mouse Tg which can be recognized by autoreactive T cells, and furthermore, these epitopes differ from those reacting with the majority of autoantibodies. These observations are what one might expect if the T-cell lines are T helper cells, since such cells specific for foreign protein antigens have been shown to recognize different epitopes from B cells specific for the same protein (Sercarz & Metzger, 1980). Further characterization of the epitopes stimulating the autoreactive T cells is currently underway using proteolytic fragments of Tg and cloned T cells.

Maron *et al.* (1983) have described autoreactive murine T-cell lines specific for Tg which were capable of eliciting thyroid lesions in normal syngeneic mice. In a number of experiments following the protocol of Maron *et al.* (1983), the cell lines described here consistently failed to induce thyroid lesions in normal recipients (data not shown). However, preliminary evidence has indicated that a small percentage of irradiated (500 rads) recipients of pre-activated thyroglobulin-specific T-cell lines can develop moderate thyroid lesions. If confirmed, this result might suggest a role for endogenous suppressor mechanisms in intact mice which act to prevent activity of the autoreactive T cells. Alternatively, damage to the thyroid epithelium such as is caused by irradiation (Penhale *et al.*, 1976) may be required before Tg-reactive T cells can infiltrate the thyroid gland. The discrepancy between our observation and those of Maron *et al.* (1983) may be significant, since different protocols were used for growing the T-cell lines. Repeated antigenic stimulation in the absence of added IL-2 appears to select for T helper cells which do not directly produce thyroid lesions, whereas using IL-2 to expand antigen-acti-

vated cells may select for a different cell type (e.g. cytotoxic T cells). It is also possible that the injected Tg-specific line cells became trapped in other tissues and thus failed to reach the thyroid. This phenomenon has been observed in the experimental autoimmune encephalomyelitis system, since only a small percentage of injected myelin basic protein-specific line cells actually accumulated in the brain, the majority being trapped by the liver and spleen (Naparstek *et al.*, 1983). Studies are now in progress to investigate the localization of line cells in recipient mice.

ACKNOWLEDGMENTS

We thank Miss N. S. Jaura for typing the manuscript. This work was supported by the Medical Research Council of Great Britain.

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